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(71) Applicant *(for all designated States except US)*: OMRIX BIOPHARMACEUTICALS S.A. [BE/BE]; 140, avenue Louise, B2, B–1050 Brussels (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: LIEBERMAN, Oded [IL/BE]; OMRIX Biopharmaceuticals S.A., 140, avenue Louise, B2, B–1050 Brussels (BE). NUR, Israel [IL/BE]; OMRIX Biopharmaceuticals S.A., 140, avenue Louise, B2, B–1050 Brussels (BE). SMITH, Andy [US/BE]; OMRIX Biopharmaceuticals S.A., 140, avenue Louise, B2, B–1050 Brussels (BE).

(74) Agents: MEYERS, Hans–Wilhelm et al.; P.O. Box 102241, D–50462 Cologne (DE).

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(54) Title: FIBRINOGEN CONCENTRATE FROM HUMAN PLASMA

(57) Abstract

A fibrinogen concentrate wherein the concentration of fibrinogen is less than 80 % of the total protein concentration and other naturally occurring plasma proteins such as fibronectin, factor VIII, von Willebrand factor, factor XIII, vitronectin, are present in amounts of at least 20 % of the total protein concentration.

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Fibrinogen concentrate from human plasma

Background of the invention

There is a recognized need for surgical adhesives and hemostatic agents for use in surgical applications. This need has been answered by the appearance of fibrin sealants produced from naturally occurring components of human blood plasma and is the subject of many patents and applications, such as WO 86/01814, EP 0 305 243 (Immuno), and US 5,605,887. These fibrin sealants are composed of the combination of a fibrinogen containing concentrate with an activating thrombin component.

Most of the existing fibrinogen containing preparations contain a limited number of plasma proteins that are separately purified and then recombined into a final product, as described in (Immuno Patent), containing primarily fibrinogen and factor XIII. Others rely on the fact that factor XIII copurifies with the fibrinogen to result in a product containing primarily fibrinogen, with trace amounts of factor XIII and fibronectin, as is described in EP 0 305 243 and in US 5,605,887. These fibrinogen concentrates as described contain 90 to 95% of the total protein present as fibrinogen. The disadvantage of this type of fibrinogen concentrate is that the clot formed, although having a high tensile strength, is very brittle and does not readily adhere to the tissues being treated. Thus, the effect as a hemostatic agent is reduced as the surface tends to re-bleed after application of the glue.

Other applications have described the production of a single donor cryoprecipitate unit from plasma and the combination of it with thrombin to for a fibrin glue (WO 86/01814). The drawbacks of such a preparation are the relatively low concentration of protein in the fibrinogen concentrate, being less than 22 g/l, and the lack of any virus inactivation technique possible to be applied to the product.

The present invention describes a fibrinogen concentrate that contains multiple proteins in high concentrations that give a surprisingly beneficial effect to the resulting fibrin glue that is produced. In the preferred presentation, the fibrinogen concentrate of the invention is virus inactivated to reduce the risk of transmission of pathogenic viruses.

Summary of Invention

The fibrinogen concentrate of this invention provides for the production of a fibrin glue that has superior qualities compared to other products in terms of hemostatic effect and reduction ensuing tissue adhesions following application of the glue. The superior effect of the glue is also evidenced in the reduction of the amount of glue required to achieve the hemostatic effects.

Accordingly, the fibrinogen concentrate is prepared from human plasma, preferably by the use of a cryoprecipitation step, wherein a multitude of proteins are concentrated, virus inactivated, and presented in a final form allowing the combination with a thrombin component for the formation of a fibrin glue. The resulting fibrinogen concentrate contains a total protein concentration of at least 30 mg/ml up to 120 mg/ml, preferably in the range of 50 to 100 mg/ml. This represents the protein concentration of the product either in the final form for a liquid product, or after reconstitution for a lyophilized product.

The fibrinogen concentrate of this invention contains multiple proteins in addition of fibrinogen, fibronectin, and factor XIII, specifically included are von Willebrand factor, factor VIII, and vitronectin, in addition to other naturally occurring plasma proteins with molecular weights greater than 30,000 kD, and which may include in particular certain growth factors. These other natuarlly occurring plasma porteins have been unexpectedly found to provide the superior qualities of the resulting fibrin glue when the total content is found to exceed 20% of the total content of proteins. That is, the superior results have been found when the concentration of fibrinogen in the fibrinogen concentrate is less than 80% of the total content of proteins, and the difference is made up of other naturally occurring plasma proteins.

This fibrinogen concentrate may be virus inactivated, preferably using the solvent detergent inactivation technique, and the most preferred presentation the concentrate is doubly inactivated by the combination of the solvent detergent method with a second virus inactivation method, for example, with pasteurization or inactivation with UVC light.

The resulting concentrate may be combined with a thrombin product for the production of a fibrin glue and for use in surgical applications.

Detailed Description of the Invention

This invention provides for a fibrinogen concentrate from human blood plasma that, when utilized in combination with a thrombin component, is highly effective as a hemostatic agent, a tissue sealant, and a tissue adhesive for use in local surgical applications. The concentrate is produced from pooled human blood plasma, is treated by one or several virus

inactivation techniques, and is suitable for routine manufacture.

The method of production of said concentrate allows the retention of most major protein components of human plasma above the molecular weight of 30,000 kD. The surprising result of this invention is that the fibrinogen concentrate produced by this method, when combined with a thrombin component, yields a superior fibrin glue product. It is the fact that these other protein components are maintained in relatively high concentrations and in biologically active forms that results in the superior qualities of the resulting glue product. The novel composition of the fibrinogen concentrate, containing less than 80% of total proteins as fibrinogen and the rest as other proteins, has resulted in the surprising qualities of this product.

The fibrinogen product is produced from human blood plasma. The fibrinogen is precipitated from the pooled plasma by one of the standard precipitation techniques, such as cryoprecipitation, alcohol precipitation, and PEG precipitation. In the preferred preparation, the fibrinogen is concentrated by a cryoprecipitation step, which also concentrates a multiple of other plasma proteins in the precipitate. The resulting precipitate is resuspended in a buffer solution and treated with aluminum hydroxide for the removal of vitamin K dependant proteins. The resulting solution is then virus inactivated by the solvent detergent inactivation technique, and the virucidal agents are removed. The resulting solution may then be concentrated on an ultrafiltration membrane, stabilized, and then formulated into a final product. This fibrinogen concentrate may be stored as a liquid, either in a frozen form or at refrigerated temperatures, or may be lyophilized.

In the preferred presentation, the solution following the SD inactivation step is treated with a second virus

inactivation process which could be pasteurization in solution in the presence of stabilizers, or by irradiation with UVC light. The product is then concentrated on an ultrafiltration membrane, stabilized, and then formulated into a final product. This fibrinogen concentrate may be stored as a liquid, either in a frozen form or at refrigerated temperatures, or may be lyophilized.

The resulting fibrinogen concentrate from this process has a final protein concentration of 30 to 120 mg/ml. In a preferred embodiment, the total protein concentration is between 50 to 100 mg/ml, and more preferred the protein concentration is about 60 to 80 mg/ml.

In the latter embodiment, the fibrinogen concentration is approximately 40 to 60 mg/ml, or about 50 to 80% of the total protein concentration of the final product, fibronectin concentration of the preferred presentation is 5 to 15 mg/ml, von Willebrand concentration is 40 to 60 IU/ml, factor XIII concentration is 2 to 10 IU/ml, factor VIII concentration is 10 to 25 IU/ml and vitronectin concentration is 0.03 to 0.07 mg/ml.

This fibrinogen concentrate may be stabilized with a variety of amino acid combinations, preferably utilizing amino acids other than α-amino acids, such as tranexamic acid and ϵ-aminocaproic acid. These stabilizers may be used in combination with other amino acids as stabilizers, preferably basic amino acids such as lysine and arginine. In the most preferred presentation, tranexamic acid and arginine are used as stabilizers.

When the fibrinogen concentrate of this invention is combined with a thrombin concentrate, a superior fibrin glue results. This is demonstrated by the use of a rabbit model for a surgical resection of a liver lobe. After excision of lateral lobe of the liver, the cut surface is treated with

a fibrin glue product and the amount of time to achieve hemostasis is recorded. The amount of glue required is also measured. In direct comparison with other commercially available glue products, that have the fibrinogen component containing > 80% of the total protein as fibrinogen, the fibrin glue of this invention was found to have dramatically reduced bleeding times of 45 seconds vs. > 195 seconds, and dramatically reduced quantities of glue required to achieve this effect of 3.6 ml vs. 4.5 to 10 ml. The competitor products evaluated were Tissucol (Immuno AG), Beriplast (Centeon), and Biocol (LFB, France).

A further unexpected benefit of the present invention is the reduction of adhesion of other tissues to the application site of the glue following the surgical operation performed above in comparison to the competitor products. Seven days following the treatment of the animals with the glue products, a necropsy was performed to evaluate the adherence of other surrounding tissues to the glue application site. The competitor products had on average at least six adhesions, while the product of this invention had less than one.

As shown above, the unexpected results of the fibrinogen concentrate clearly demonstrate the superiority of this product to other commercially available products in terms of hemostatic effect, ability to adhere to the cut surface, reduction in quantity of glue required, and ability to reduce adhesions to surrounding tissues. These beneficial qualities of the product show it to be useful for use in any surgical applications related to hemostasis, tissue adhesion, and tissue sealing.

Examples

The following examples are representative of the practice of the invention.

Example 1

Composition of the Fibrinogen Concentrate

The fibrinogen concentrate had the following composition due to biochemical analysis.

Total protein - 69 mg/ml
Fibrinogen (clottable) - 48 mg/ml
Fibronectin - 12 mg/ml
Vitronectin - 0.05 mg/ml
von Willebrand Factor - 53 IU/ml
Factor XIII - 5 IU/ml
Factor VIII - 17 IU/ml

Example 2

Results of Rabbit Model for Liver Resection

The fibrinogen concentrate of this invention, indicated as Quixil below, was compared in the rabbit model for liver resection against commercially available products. The results are as follows:

Product	Bleeding Time (seconds)	Quantity used (ml)	Number of Adhesions
QUIXIL	44	3.2	0.8
BERIPLAST	197	4.5	5.7
TISSUCOL	255	9.5	7.6
BIOCOL	240	10.0	not done

Example 3

Characterization of the Fibrinogen Concentrate

Introduction: The purpose of this study was to both identify and quantify the active component, fibrinogen, and other protein components present in the fibrinogen concentrate. In order to achieve these goals, samples from 7 different batches of fibrinogen concentrate were subjected to gradient SDS polyacrylamide gel electrophoresis (SDS PAGE) (Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, 1989. 3:18.47-18.55). To identify the proteins, the molecular weights of the reduced proteins were analyzed according to their relative migration on a SDS reducing gel. To assess the relative amounts of fibrinogen concentrate as compared to other proteins, densitometric scanning of the bands was performed.

Method: Samples of fibrinogen concentrate batches and low molecular weight (LMW) marker (BIORAD) were run by SDS gradient PAGE. Fibrinogen concentrate samples were diluted 1:100 in double distilled water, and 20 μl of the diluted samples (10-20 μg protein) as well as 20 μl LMW marker were applied to a 5-15 % gradient polyacrylamide denaturing gel. The samples were run overnight at 15 mA, and the gel was stained with Coomassie Blue. The molecular weights of the protein bands were calculated according to their relative migration vs. molecular weight from the low molecular weight marker. Densitometric scanning of the protein bands was performed using a densitometer (Molecular Dynamics) and analyzed by the Imagequant program.

Results: Fig. 1a depicts the SDS PAGE of the fibrinogen concentrate samples. The 3 strongest bands at 88, 75 and 68 kD, correspond to the fibrinogen 4,22 α-A, Bβ and γ chains, respectively. The same is true for the high molecular weight bands, corresponding to von Willebrand and fibronectin. In more detail, the Fig. 1 shows SDS-PAGE Analysis of Fibrinogen Concentrate Proteins. (A) SDS-PAGE analysis - SDS-PAGE was carried out as described in Materials and Methods. Lane 1

through 5, 7, and 8: Fibrinogen Concentrate Samples; Lane 6: LMW marker (BIORAD) with 112, 84, 53.2, 34.9, 28.7, and 20.5 kD standards. Bands denoted A correspond to high molecular weight von Willebrand and fibronectin proteins, B correspond to fibrinogen α-A, Bß and γ chains, and C+D correspond to low molecular weight proteins. (B) Densitometric scans of bands from SDS-PAGE - Densitometry was carried out as described in Materials and Methods. Graphs 1,2,3,5,7 and 8 correspond to the scans of bands from the corresponding lanes in (A). The letters denoting the bands in (B) are used to denote their corresponding peaks in (B).

Table 1 summarizes the molecular weights of all the bands in each sample calculated from the linear regression curve made according to the distance of migration of the LMW marker bands. Fig. 1b depicts the superimposed results of the densitometric scanning of the bands from each lane. The peak areas show that the relative amounts of von Willebrand and fibronectin (15-20 % of total protein), fibrinogen (75-80 % of total protein), and low molecular weight proteins (5 % of total protein) are the same in all batches of fibrinogen concentrate.

Fibrinogen Concentrate	
Peak #	Mean MW (D)
1	159,224
2	88,163
3	74,512
4	67,849
6	44,964
7	33,470

Table 1: Molecular weights of fibrinogen concentrate proteins analyzed by SDS-PAGE. The molecular weight of each protein band was derived by interpolation from a linear regression of Log MW vs. Distance from the bands corresponding to the LMW marker.

Conclusion: SDS PAGE analysis of the fibrinogen concentrate demonstrates that the final product consists of two high molecular weight polypeptides. Von Willebrand and fibronectin (comprising 15-20 % of the total protein), of the fibrinogen α-A, Bß and γ chains (comprising 75-80 % of the total protein), and low molecular weight polypeptides (comprising 2-10 % of the total protein).

Claims:

1. A fibrinogen concentrate wherein the concentration of fibrinogen is less than 80% of the total protein concentration and other naturally occurring plasma proteins such as fibronectin, factor VIII, von Willebrand factor, factor XIII, vitronectin, are present in amounts of at least 20% the total protein concentration.

2. The fibrinogen concentrate according to claim 1 where the total protein concentration is 30 to 120 mg/ml.

3. The fibrinogen concentrate according to claim 1 which is produced from pooled human blood plasma and/or precipitate of human blood plasma.

4. The fibrinogen concentrate according to claim 3 which is produced from a cryoprecipitate of human blood plasma.

5. The fibrinogen concentrate according to claim 1 which contains three or more plasma proteins in combination.

6. The fibrinogen concentrate according to claim 5 which contains fibrinogen, fibronectin, and factor XIII and/or other proteins.

7. The fibrinogen concentrate according to claim 6 which also contains von Willebrand factor, factor VIII, vitronectin or combinations thereof.

8. The fibrinogen concentrate according to claim 6 in which the other proteins included are therapeutic agents and/or growth factors.

9. The fibrinogen concentrate according to claim 1 comprising antifibrinolytics, such as tranexamic acid and/or ε-aminocaproic acid.

10. The fibrinogen concentrate according to claim 1 where the product is virus inactivated.

11. The fibrinogen concentrate according to claim 10 where the virus inactivation is performed using the solvent detergent technique.

12. The fibrinogen concentrate according to claim 10 where an addition virus inactivation step of pasteurization or UVC irradiation is performed.

13. The fibrinogen concentrate according to claim 1 which comprises stabilizers, such as amino acids.

14. The fibrinogen concentrate according to claim 13 where the amino acids used include tranexamic acid, ε-aminocaproic acid, arginine, lysine or combinations thereof.

15. A two component fibrin glue comprising as one component the fibrinogen concentrate according to one of the claims 1 to 14 and as second component an agent which is capable to form fibrin from fibrinogen, such as thrombin or fractions of snake venoms.

16. Use of a fibrinogen concentrate according to claim 1 for the manufacturing of a fibrin glue for application in local surgical applications for wound healing, sealing and hemostasis.

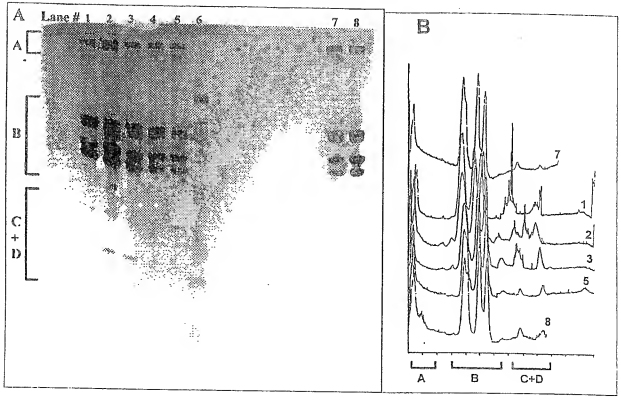

Fig. 1

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/36 A61L25/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 305 243 A (CENTRE REGIONAL DE TRANSFUSION SANGUINE DE LILLE) 1 March 1989 cited in the application see the whole document	1-16
A	EP 0 534 178 A (OCTAPHARMA AG) 31 March 1993 see the whole document	1-16
A	US 5 605 887 A (PINES E. ET AL.) 25 February 1997 cited in the application see the whole document	1-16
A	US 5 420 250 A (LONTZ J.F.) 30 May 1995 see the whole document	1-16

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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